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Immunohistochemical Distribution of Ferritin, Lactoferrin, and Transferrin in Granulomas of Bovine Paratuberculosis

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Granulomatous lesions of bovine paratuberculosis contained ferritin, lactoferrin, and a small amount of transferrin, as demonstrated by the immunohistochemical method. Macrophages in the normal bovine ileum did not contain lactoferrin and transferrin; however, ferritin was found in individual macrophages of Peyer's patches. These results may help elucidate the relationship between intracellular growth of *Mycobacterium paratuberculosis* and the presence of iron-binding proteins in the granulomas.

Paratuberculosis caused by *Mycobacterium paratuberculosis* is one of the most important chronic infectious diseases in the cattle industry worldwide (5). The disease is characterized pathologically by granulomatous enteritis and clinically by intermittent diarrhea and progressive emaciation (3, 5).

The lack of an accurate and sensitive diagnostic test makes it difficult to control the spread of the disease (5). Another problem is the extremely long incubation period, 2 to 4 years. Therefore, it is important that the pathogenesis of this disease be elucidated.

M. paratuberculosis is mycobactin dependent and grows very slowly in artificial medium (up to 3 months) (1, 5, 15). Moreover, the organisms reside silently in the mononuclear phagocytic system (MPS) cells of the host for a long period. However, following the clinical stage, they show vigorous growth in the MPS cells of the granulomas (3, 5), and more than 10^8 organisms per gram are shed in the feces (5).

The exact mechanism leading to the difference in bacterial growth is important for understanding the pathogenesis, but it is poorly defined. Iron is essential for bacterial growth and metabolism (4, 6, 12). The different growth rate of the organisms in macrophages cultured with and without iron suggests that intracellular growth is regulated by the iron concentration (21). Mycobacteria produce the iron-binding components mycobactin (15) and exochelin (1, 10, 11), which aid in acquiring iron from the extracellular environment. *M. paratuberculosis* is thought to acquire iron from host iron-binding proteins, because the bacterial iron-binding compound has been found to acquire iron from the iron-binding protein in in vitro studies (1, 8, 10, 11). Only scant evidence for the presence of iron-binding proteins in the MPS cells of granulomas in animals and humans has been reported. Accordingly, we examined whether paratuberculous granulomas in cattle contain the major iron-binding proteins ferritin (FT), lactoferrin (LF), and transferrin (TF) by using the immunohistochemical method.

Infected ileums were collected from 20 cattle diagnosed as having paratuberculosis. The cattle were from 2 to 11 years old and included 14 Holstein, 4 Japanese brown, and 3 Aberdeen-Angus breeds. The diagnosis was obtained by complement fixation tests, microscopic examination for acid-fast bacilli in fecal smears, and clinical observation. Johnin intradermal tests were also carried out, but all animals proved to be negative after 48 h (see Table 1).

Control intestine samples were collected from 10 healthy Holstein cattle, 2 to 4 years old, which were born and raised at the Hokkaido National Agricultural Experimental Station. The herd was known to be free from paratuberculosis.

M. paratuberculosis was isolated in 9 of 20 cases by culture in Herrold egg yolk medium. The acid-fast isolates were identified by their mycobactin dependency.

After macroscopic examination, the intestine samples were fixed in 10% buffered Formalin for 7 to 60 days, after which four 4-μm-thick serial sections were cut. The sections were stained with hematoxylin and eosin, Ziehl-Neelsen, and Berlin blue.

For light-microscopic demonstration of iron-binding proteins, the peroxidase-antiperoxidase (PAP) method of Sternberger et al. (16) was used. Serial sections were mounted on slides precoated with 0.1% Neoplen (mesh cement for electron microscopy; Nissin EM Ltd., Tokyo, Japan). The sections were then dewaxed in xylene and hydrated, incubated in 0.1% trypsin (trypsin 1:250; Difco Laboratories, Detroit, Mich.), and washed in three 10-min changes of cold phosphate-buffered saline (PBS). Washing with PBS was carried out in the same manner in the succeeding steps. Endogenous peroxidase was blocked by immersing the sections in methanol containing 0.03% hydrogen peroxide for 30 min at room temperature and by washing with PBS. The sections were treated with normal goat serum, diluted in 1/10 PBS for 30 min, and then drained. Specific rabbit anti-bovine iron-binding protein serum was applied for 30 min at room temperature, and the sections were left overnight at 4°C and washed with PBS. The sections were incubated with goat anti-rabbit immunoglobulin G (IgG) (Cappel Laboratories, Cochranville, Pa.) for 30 min at room temperature and then washed with PBS. They were incubated with rabbit PAP (Cappel) for 30 min, washed with PBS for 30 min, and immersed in Tris-buffered saline, pH 7.6. The sections were incubated with serum in a moist chamber for all steps. Finally, 0.05% 3,3'-diaminobenzidine in 0.01% hydrogen peroxide in Tris-buffered saline was applied for 5 to 10 min. The sections were washed gently in running tap water for 5 min and then stained with Ziehl-Neelsen stain. They were counterstained with 1% methyl

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TABLE 1. Clinical, bacteriologic, and immunologic findings

Case no.	Breed[a]	Age (yr)	Diarrhea	Johnin test[b]	CF titer[c]	Fecal smear[d]	Bacteria isolated
J-2	H	5	+	−	20	+	ND[e]
J-3	B	2	+	ND	ND	ND	ND
J-4	A	6	+	−	40	+	+
J-5	H	6	+	−	10	+	+
J-8	A	7	+	ND	10	+	+
J-9	H	7	−	−	10	+	+
J-10	A	5	+	−	40	+	+
J-11	H	4	+	−	10	+	ND
J-14	H	6	+	−	80	+	ND
J-16	H	6	+	−	20	+	ND
J-18	B	11	−	−	ND	+	+
J-19	H	4	−	−	10	−	ND
J-21	H	3	+	−	40	+	+
J-22	B	3	−	−	10	−	+
J-23	B	6	+	−	20	+	ND
J-24	H	8	+	−	20	+	ND
J-27	H	3	+	−	20	+	+
J-28	H	2	−	−	20	−	ND
J-29	H	5	+	ND	40	+	ND
J-30	H	3	+	−	40	+	ND

[a] H, Holstein; B, Japanese Brown; A, Aberdeen-Angus.
[b] Johnin intradermal tests for paratuberculosis after 48 h. −, No reaction.
[c] Complement fixation (CF) test titers for paratuberculosis.
[d] +, Aggregated acid-fast bacilli demonstrated; −, bacilli not demonstrated.
[e] ND, Not done.

green in 0.1 M acetic buffer, pH 4, for 15 min, dehydrated, cleared, and mounted in Eukitt (O. Kindler, Federal Republic of Germany).

Bovine FT was prepared from bovine spleen as previously described (7). Anti-bovine FT serum was obtained from rabbits and purified by affinity chromatography as previously described (7).

Anti-bovine LF rabbit serum was prepared with purified bovine LF (provided by Central Laboratory, Morinaga Dairy Industry Co. Ltd., Tokyo, Japan) as previously described (14, 17).

Purified bovine TF was supplied by J. H. Block, University of Glasgow, Scotland. Purified bovine TF (3.7 mg) in 1 ml of PBS was emulsified with 1 ml of Freund incomplete adjuvant (Difco) and 1 mg of Muramyl Dipeptid (a synthetic preparation provided by A. Kuwano, Daiichi Pharmaceutical Co. Ltd., Tokyo, Japan). One milliliter of the emulsion was injected subcutaneously into a rabbit at 1, 2, and 3 weeks and intramuscularly 2 weeks later. Antiserum was obtained 5 days after the final immunization. The specificity of the antibody was examined by the immunodiffusion test. The antiserum formed a single band against purified bovine TF and normal bovine serum. Preliminary immunohistochemical staining showed that most of the antisera, except the anti-TF serum, contained various concentrations of antimycobacterial antibody. A 4-ml amount of each antiserum was absorbed by 1 g of packed wet *Mycobacterium phlei* ATCC 354 cells (heat killed and washed with PBS) after 1 h at room temperature.

To evaluate the specificity of the reaction, the following modifications of the staining processes were performed: (i) the primary antiserum was omitted or replaced by normal rabbit serum; (ii) goat anti-rabbit IgG was omitted; (iii) PAP was omitted.

M. paratuberculosis was isolated from eight ileal samples examined (Table 1). All ileal sections revealed typical paratuberculosis granulomas consisting of epithelioid and giant cells. The size, predominant cell type, and number of acid-fast bacilli in the granulomas varied (Table 2). No changes other than the granulomas were observed in the sections. A weakly diffuse Berlin blue reaction was observed in the lesions, especially in the submucosal granulomas. Normal control ileums did not show any histopathological changes.

Triple staining with PAP, Ziehl-Neelsen, and methyl green provided a good contrast, so that the distribution of iron-binding proteins, acid-fast bacilli, and cell types was easily recognized.

In normal ileums, a weak FT staining in individual macrophages of Peyer's patches was observed (Fig. 1a). Only a few macrophages in the lamina propria mucosae showed a faint cytoplasmic reaction. Reaction to LF was found in the neutrophilic granules (Fig. 1b); however, no MPS cells reacted to LF. Various degrees of the reaction to TF were observed in the intercellular spaces and in the vessels (Fig. 1c). The MPS cells did not react to TF.

In 12 of 21 paratuberculous ileums (Table 2), the epithelioid and giant cells of the inflammatory granulomas were positive for FT (Fig. 2 and 3). The cytoplasmic reaction was diffuse; however, some differences in intensity were found in the same granuloma. In the four cases of widespread epithelioid granulomas, the reaction in the submucosa was generally stronger than in the lamina propria mucosae.

Epithelioid and giant cells in the granulomas and neutrophils of 12 of 20 cases showed LF reactions similar to those observed for FT (Fig. 4 through 6). However, no specific reaction was demonstrated in the other cases. In contrast to the FT reaction, granulomas reacted more strongly in the lamina propria mucosae than in the submucosa. In the small giant-cell granulomas, the peripheral cytoplasm tended to

TABLE 2. Histopathologic and immunohistochemical findings

Case no.	Histology		Immunohistochemistry[c]		
	Predominant granuloma cell type[a]	Bacterial index[b]	FT	LF	TF
J-2	E	1+	−	−	−
J-3	E	1+	+	+	+
J-4	E	2+	−	−	−
J-5	E	2+	+	+	−
J-8	E	2+	+	+	+
J-9	E	2+	−	−	−
J-10	E	3+	+	+	+
J-11	E	3+	−	−	−
J-14	E	3+	+	+	−
J-16	E	1+	−	−	−
J-18	E + G	2+	+	+	−
J-19	M	1+	−	−	−
J-21	G	1+	+	+	−
J-22	G	1+	+	+	−
J-23	G	1+	−	−	−
J-24	G	1+	−	−	−
J-27	G	1+	+	+	+
J-28	G	1+	+	+	+
J-29	G	3+	+	+	−
J-30	G	3+	+	+	−

[a] E, epithelioid cells; E + G, epithelioid and giant cells; M, macrophages (immature epithelioid cells); G, giant cells.
[b] Number of bacteria and severity of infection: 1+, mild; 2+, moderate; 3+, marked.
[c] Presence (+) or absence (−) of FT, LF, and TF.

show a stronger reaction (Fig. 5). Epithelioid and giant cells in the granulomas of 5 of 21 cases (Table 2) had a much weaker reaction to TF than to FT and LF (Fig. 7). The reaction to TF was not improved by prolonged incubation or by increasing the concentration of antiserum. In the widespread granulomas, submucosal lesions showed a weaker TF reaction than did the lamina propria mucosae. In the control experiments, there was no reaction, confirming the specificity of the reaction.

In the present study, we demonstrated that MPS cells in bovine paratuberculous granulomas contained FT, LF, and very small amounts of TF. In contrast, MPS cells in the normal ileum did not react to LF and TF; however, FT was found in some MPS cells there.

Examination of sections from eight infected ileums which did not stain for any iron-binding protein suggested that the loss of antigenicity might have been due to delay between collection of samples and fixation or to excessive fixation.

Intracellular ferric iron in the bovine paratuberculous granulomas was observed by Lepper and Wilks (9) with the Berlin blue reaction. Their results suggested that macrophages in ileocecal valves, where the granulomas first occur, contained much iron and many bacilli and that well-differentiated epithelioid and giant cells contained little or no iron and fewer bacilli. Contrary to their study, we observed iron-binding proteins in the differentiated MPS cells regardless of the number of bacilli.

Experiments with mice on iron-depleted or -deficient diets revealed that high levels of intracellular iron favor growth of the bacilli in tissues (9).

The importance of iron for mycobacterial growth was reported for isolated macrophages as well. In macrophages from cattle (2) and sheep (21) cultured in the usual medium, it was demonstrated that although the bacilli were not destroyed, continuous growth did not occur. Adding iron or other factors to the medium augments intracellular growth (21).

Iron is essential for bacterial growth and metabolism (4, 6, 12). Therefore, mycobacteria produce two types of iron-binding compounds, mycobactin and exochelin, to acquire iron from the environment (host MPS cell and medium) through their thick, lipid-rich boundary layers. Mycobactin is a lipophilic molecule and is located in the boundary layers of organisms, where it transports iron across the thick lipoidal layers of the mycobacterial cell (11). Exochelin is an extracellular, water-soluble iron-binding compound (1, 10, 11). Mycobactin and exochelin can acquire iron from FT and, presumably, TF (1, 8, 10, 11) in vitro. Exochelin is suspected to acquire iron from LF (C. Ratledge, personal communication). Therefore, we believe that the iron-binding proteins demonstrated in the present study have an important role in growth.

Van Snick et al. (18–20) demonstrated that the mechanism of inflammatory hyposideremia is mediated by the iron-binding proteins LF, TF, and FT. In their studies, MPS cells (monocytes) ingested LF, which was produced by neutrophils (13), and transferred iron from LF to FT. The present findings suggest that infectious hyposideremia could occur at any time in the course of the disease. We suspect that when the MPS cells containing a few *M. paratuberculosis* cells start to synthesize FT from ingested LF or other iron compounds, the organisms can acquire sufficient iron for minimum growth.

Once the initial impediment to growth has been overcome, the bacilli are able to produce their own exochelins and, thus, elaborate a mechanism for iron acquisition (1). The

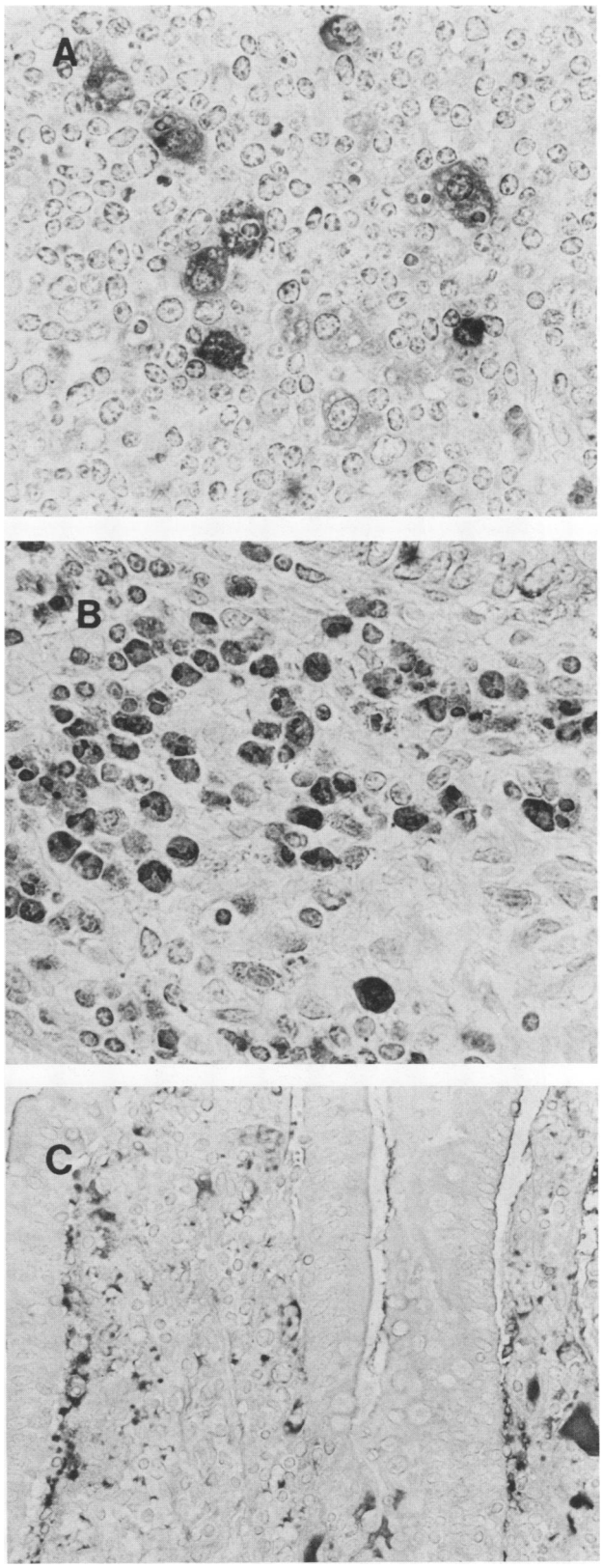


FIG. 1. Iron-binding proteins in normal ileums. (A) Ferritin in the macrophages in a Peyer's patch. Magnification, ×520. (B) Lactoferrin in neutrophils in the lamina propria mucosae (×600). (C) Transferrin in intercellular spaces of lamina propria mucosae (×520).

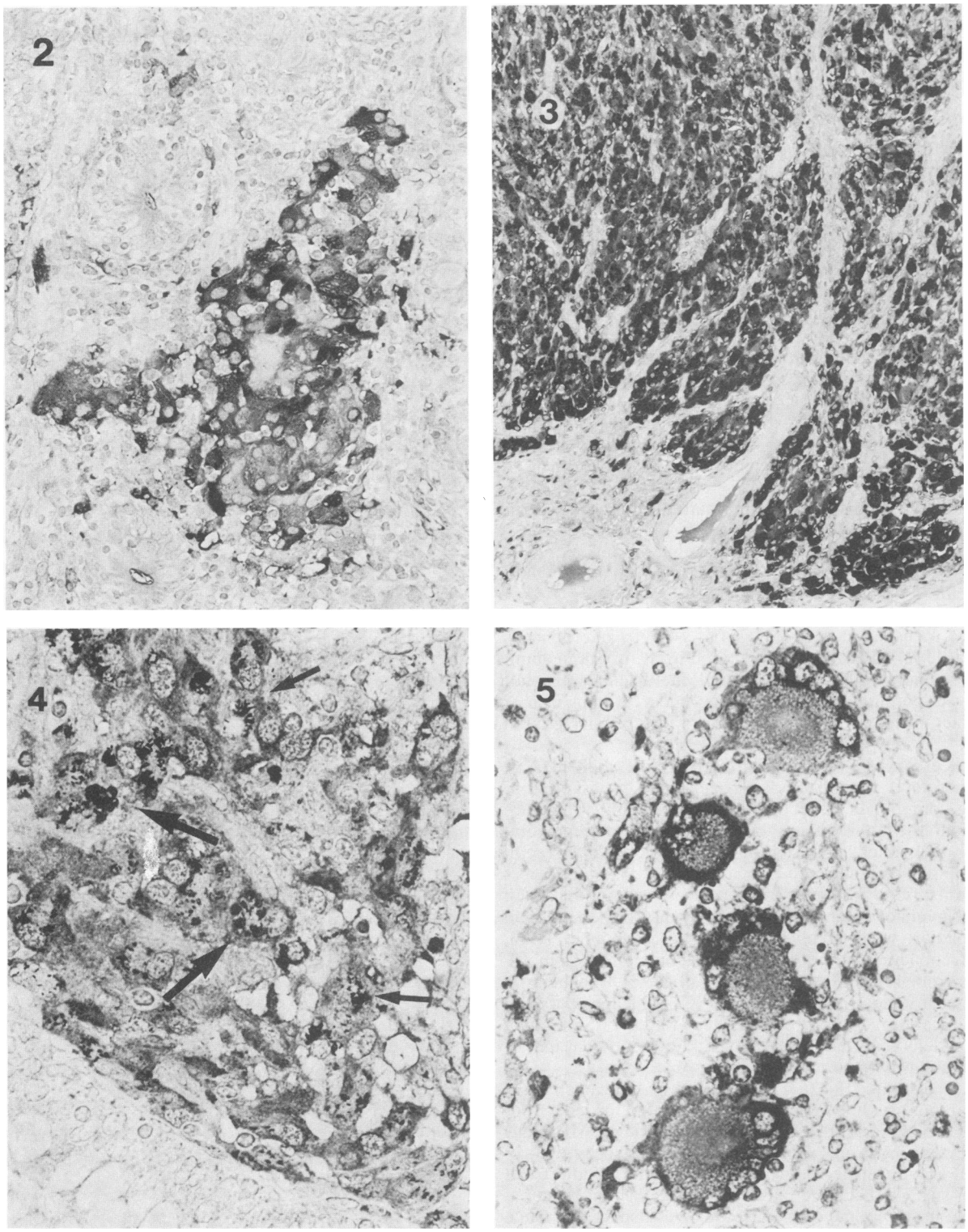
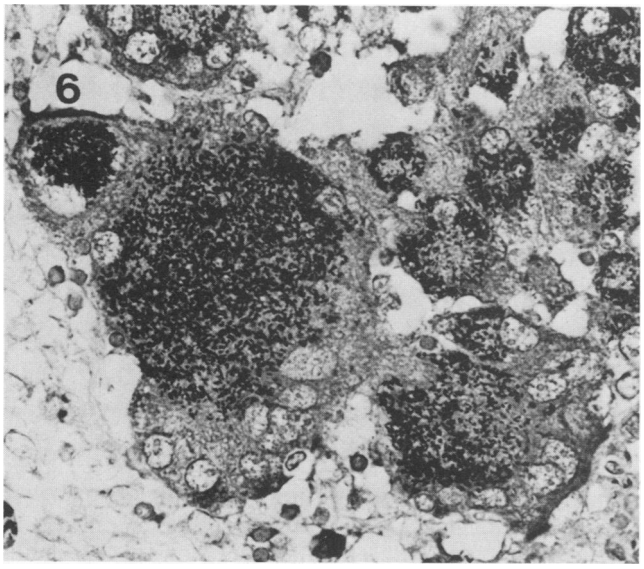


FIG. 2–5. 2. Staining for ferritin in a paratuberculous granuloma. Epithelioid and giant cells stained in various degrees (×260). 3. Staining for ferritin in the granulomas in the tunica submucosae. Note the negative reaction in the connective tissue and vessels (×130). 4. Staining for lactoferrin in the epithelioid cell granuloma in the lamina propria mucosae. Clumps of acid-fast bacilli (arrows) are present in the cytoplasm (×520). 5. Staining for lactoferrin in the lamina propria mucosae giant cells which contain a few acid-fast bacilli. Strong reaction is found in the peripheral cytoplasm (×520).



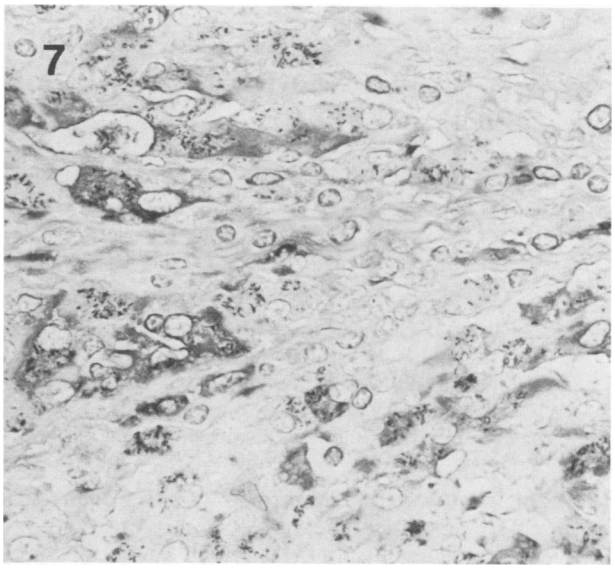


FIG. 6 and 7. 6. Staining for lactoferrin in the giant cells which contain numerous acid-fast bacilli (×520). 7. Staining for transferrin in the epithelioid cell granuloma (×520).

bacilli may not need more intracellular iron in the later stage than in the initial stage of growth. The fact that intracellular concentrations of FT and LF did not correlate well with the number of bacilli in the present study may support this hypothesis. In conclusion, our results support the hypothesis that *M. paratuberculosis* can grow in vivo in the absence of an external supply of mycobactin (1).

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